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PLASMODIUM FALCIPARUM ANTIGENS AND THEIR VACCINE AND DIAGNOSTIC APPLICATIONS

CONTEXT OF THE INVENTION

a) Field of the invention

The present invention relates to novel *Plasmodium falciparum* antigens and to their vaccine and diagnostic applications. More particularly, the present invention relates to polypeptide molecules and immunogenic polynucleotide, to compositions comprising them, and to methods for diagnosis of and vaccination against malaria.

b) Brief description of the prior art

Malaria is a disease caused by infection of protozoic parasites belonging to apicomplexes of the species *Plasmodium* and transmitted by female mosquitoes of the genus *Anopheles*. Despite the fact that since 1998, the WHO has classified malaria as one of the three infectious diseases of major interest to world health, alongside tuberculosis and AIDS, there is still no effective vaccine against this disease.

Previous studies have determined antigenic polypeptides for the pre-erythrocytic stage of the disease, in particular SALSA (Sporozoite Liver Stage Antigen) polypeptides described in European patent EP-A-0 407 230, LSA 1 (Liver Stage Antigen) polypeptides described in International patent application WO 92/13884 and LSA-3 described in French patent FR 2 735 478.

The present invention relates to novel polynucleotide and polypeptide molecules specific to the pre-erythrocytic stages and to their use as an active principle for an anti-malaria vaccine or in methods for diagnosing the disease.

SUMMARY OF THE INVENTION

Applicant has identified a series of 120 genomic DNA fragments coding for proteins expressed in the pre-erythrocytic stages, i.e., the sporozoite stage and/or the liver stage. Initial characterization of this series of clones has resulted in identification of the LSA-1 antigen, then

SALSA, then STARP, then LSA-3. More recent work on 10 fragments from the same clone library coding for pre-erythrocytic stages have provided more information concerning 8 of them; 3 have been shown to be genes that are already known to be expressed in the erythrocytic stage and the other 5 are novel genes that have not been described to date, and for which expression during the pre-erythrocytic stages has been confirmed.

Further, work carried out using cells from volunteers protected by irradiated sporozoites, cells from chimpanzees protected by the same method and cells from chimpanzees such as *Aotus trivirgatus*, protected by immunization with the antigen LSA-3, have led to a characterization of cell responses with a high level of γ-interferon secretion, generally associated with a low level of antibody production, as being associated with the protected condition, and vice-versa.

Two of the novel pre-erythrocytic genes that were studied, DG747 and DG772, have several remarkable properties: they generate cell responses with a high level of γ-interferon, detected by ELISPOT in volunteers protected by irradiated sporozoites, which are also found for several regions of the LSA-3 antigen but which are absent for 4 regions of the LSA-1 antigen, two of SALSA, two of STARP and two of the "CircumSporozoite Protein". Those two clones are also positive in the same tests in chimpanzees protected by irradiated sporozoites. The differential response profile between the protected chimpanzees and chimpanzees that received irradiated sporozoites in too high dose, and not protected, is identical to that recorded with the LSA-3 molecule which is capable of inducing protection. This response profile corresponds, according to studies carried out with the rodent, to the capacity to induce specific cell recruitment on the intra-hepatic level. The complete sequence of the two genes has been identified. The corresponding proteins have high antigenicity in individuals exposed to the parasite in an endemic zone (reaction in 80% of adults in the endemic zone). Their location on the surface of the sporozoite and their production during intra-hepatic maturation of the parasite has been confirmed by various biological methods. Their immunogenicity in the animal in the

form of recombinant proteins or in the form of plasmids (genetic immunization) has been demonstrated.

More particularly, one aspect the present invention concerns an isolated or purified polynucleotide comprising a nucleotide sequence with at least 60%, preferably at least 80% and more preferably at least 95% identity with SEQ ID NO:1 (DG747) or SEQ ID NO: 2 (DG772).

In a further aspect, the present invention concerns an isolated or purified polynucleotide comprising at least 10 consecutive nucleotides identical to SEQ ID NO:1 or SEQ ID NO: 2. The invention also concerns isolated or purified polynucleotides which hybridize under highly stringent conditions with a polynucleotide as defined above.

In a still further aspect, the present invention concerns an isolated or purified polypeptide coded by a polynucleotide as defined above. In a preferred implementation, the polypeptide of the invention has at least 60%, preferably at least 80% and more preferably at least 95% homology with SEQ ID NO: 3 (DG747) or SEQ ID NO: 4 (DG772). In a further preferred embodiment, the polypeptide of the invention comprises at least 5 consecutive amino acids identical to one of SEQ ID NOs: 3 to 8. In a still further preferred embodiment, the polypeptide of the invention has at least 40%, preferably at least 60%, more preferably at least 80% and still more preferably at least 95% identity with one of SEQ ID NOs: 3 to 8, 10 and 12. The invention also encompasses recombinant or chimeric polypeptides comprising at least one polypeptide as defined above.

In a further aspect, the present invention concerns an isolated or purified antigen consisting of a polynucleotide or a polypeptide as defined above.

In a still further aspect, the present invention concerns an antigenic conjugate constituted by a polynucleotide and/or a polypeptide as defined above; and a support onto which said polynucleotide/polypeptides are adsorbed. Said conjugate can advantageously be used to immunize individuals who have been infected or who are susceptible of being infected with malaria.

In a further aspect, the present invention concerns monoclonal or polyclonal antibodies, preferably humanized, specifically recognizing at least one of the polynucleotides, polypeptides and/or conjugates defined above. In a related aspect, the present invention concerns pharmaceutical compositions which comprise, as the active substance, one or more of said polyclonal or monoclonal antibodies in association with a pharmaceutically acceptable vehicle.

In accordance with a further aspect, the present invention concerns a cloning or expression vector (such as plasmids, cosmids or phages) comprising a polynucleotide sequence in accordance with the present invention. The invention also encompasses host cells comprising said vector, and more particularly recombinant *E. Coli* cells deposited at the C.N.C.M [National Collection of Microorganism Cultures] on 23rd May 2001 with accession numbers I-2671 and I-2672.

In a further aspect, the present invention concerns an immunogenic composition comprising polynucleotides, polypeptides and/or conjugates as defined above; and a pharmaceutically acceptable vehicle.

A further related aspect of the present invention concerns an anti-malaria vaccine comprising polynucleotides, polypeptides and/or conjugates as defined above; and a pharmaceutically acceptable vehicle. Preferably, the compositions and vaccines of the present invention are used to produce drugs intended for the prevention and/or treatment of malaria.

In accordance with a further aspect, the present invention concerns methods and kits for in vitro diagnosis of malaria in an individual who is susceptible of being infected with *Plasmodium falciparum*. In accordance with a preferred implementation, the method comprises the following steps:

a) bringing a biological tissue and/or fluid removed from an individual who is susceptible of being infected with *Plasmodium falciparum* into contact with an antibody as defined above under conditions allowing an immunological reaction to allow the formation of immune complexes; and

b) detecting the immune complexes formed *in vitro*.

In accordance with a further preferred embodiment, the diagnostic method comprises the following steps:

a) bringing a biological tissue and/or fluid removed from an individual susceptible of being infected with *Plasmodium falciparum* into contact with polynucleotides, polypeptides and/or those conjugates as defined above under conditions allowing an immunological reaction to allow the formation of immune complexes involving at least one of said elements and antibodies that may be present in said biological tissue or fluid; and

b) detecting any immune complexes that are eventually formed *in vitro*.

In accordance with a preferred embodiment, the kit of the invention for *in vitro* diagnosis of malaria comprises the following elements:

a) at least one element selected from the group formed by: polynucleotides, polypeptides and conjugates as defined above;

b) reagents for constituting a medium suitable for a binding reaction between a test sample and at least one of the elements defined in a); and

c) reagents allowing the detection of antigen-antibody complexes produced by said binding reaction, said reagents also possibly carrying a label or being susceptible of being themselves recognized by a labeled reagent.

In accordance with a further preferred embodiment, the kit of the invention comprises the following elements:

- antibodies as defined above;
- reagents for constituting a medium suitable for a binding reaction between a test sample and at least one said antibody ; and

- reagents allowing the detection of antigen-antibody complexes produced by said binding reaction, said reagents also possibly carrying a label or susceptible of being themselves recognized by a labeled reagent.

One major advantage of the present invention is that it provides novel polynucleotide and polypeptide molecules specific to the pre-erythrocytic stages of malaria. The polynucleotide and polypeptide molecules of the invention have several remarkable properties. They generate cell responses with a high level of γ-interferon. The results obtained also suggest that the polynucleotide and polypeptide molecules of the invention have the capacity to induce specific cell recruitment on the intra-hepatic level. The invention also provides effective anti-malaria vaccines and diagnostic methods sensitive to malaria.

A number of other aims and advantages of the present invention will become apparent from the following non-limiting description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, 1C and **1D** show nucleotide sequence listings (SEQ ID NOs: 1 and 2) and amino acid sequences (SEQ ID NOs: 3 and 4) of DG747 and DG772.

Figure 1E shows the degenerate repeat sequences characteristic of the DG747 clone (SEQ ID NOs: 5-8).

Figure 2A shows the gene sequence coding for DG747 (SEQ ID NOs: 9 and 10) extracted from the genome database for the 3D7 clone of *Plasmodium falciparum* (gene PfB00155). The greyed out areas (▩) show the sequence corresponding to the DG747 clone. The difference with the sequence derived from strain T9.96 (positions 344, 357) is shown in **bold** in the sequence.

Figure 2B shows the sequence of the gene coding for DG772 (SEQ ID NOs: 9 and 10) extracted from the genome database for the 3D7 clone of *Plasmodium falciparum*. The greyed out areas (▩) show the sequence corresponding to the DG772 clone. Difference from the sequence derived from strain T9.96 (position 3612) is shown in **bold** in the sequence.

Figures 3.1(a) and **3.1(b)** are diagrammatic representations of proteins corresponding to DG747 (a) and DG772 (b). The solid arrows indicate the position of primers used to study the fragment conservation. The open arrows indicate primers used in the RT-PCR reaction. 3.1(a): the hatched portion indicates a repeat region. 3.1(b): the two consensus regions 5' cys and 3' cys are shown on the gene. The dotted portion represents the assumed transmembrane regions and non-transcribed regions.

Figures 3.2A, 3.2B, 3.2C and **3.2D** show IFATs of the sporozoite and blood stages of *P. falciparum* and sporozoites of *P. yoelii* with anti-DG747 or anti-DG772 antibodies. Fig 3.2A, Fig.3.2B: sporozoite of *P. falciparum* (A) or P.*yoelii* (B) labeled with anti-747 or anti-772; Fig. 3.2C, Fig. 3.2D: asynchronous blood stage labeled for anti-747 (C) or anti-772 (D); a, t, s: ring, trophozoite or schizont forms respectively.

Figures 3.3(a) and **3.3(b)** show Western blots of *P. falciparum*, *P. yoelii* and *P. berghei* using anti-His_6-747 (a) and anti-His_6-772 (b) antibodies. Track 1: *P. falciparum* sporozoites; Track 2: *P. falciparum* blood stage, ring form; Track 3: *P. falciparum* blood stage, schizont form; Track 4: supernatant from asynchronous culture; Track 5: human red blood cells; Track 6: *P. yoelli* sporozoites; Track 7: *P. yoelii* blood stage; Track 8: *P. berghei* blood stage; 9: mouse red blood cells.

Figures 3.4(a), 3.4(b) and **3.4 (c)** show photographs of the results of PCR of the DNA from 12 different strains with specific primers for DG747 3.4(a) and DG772 3.4(b). The control, 3.4(c), is a constitutive gene, PCNA [Kilbey, 1993 #519]. The DNAs used were derived from the strains: NF54, B1, F32, D7, D25, D28, D41, D50, D51, H1, L1, Mad20, T9.96, PA (wells 1 to 14, left to right). Well 15 contains no DNA. The size of the PCR product, corresponding to that expected, is indicated to the siae of the arrows.

Figures 3.5(a) and **3.5 (b)** illustrate by means of graphs the prevalence of humoral responses against His_6-747 (a) and His_6-772 (b) in two age groups and in two different endemic zones.

Figures 3.6(a) and **3.6(b)** illustrate by means of graphs the cell responses against His_6-747 and His_6-772 in humans and chimpanzees immunized with irradiated sporozoites. Fig. 3.6a: Elispot detection of secretion of IFN-γ from cells deriving from humans immunized with irradiated sporozoites; Fig. 3.6b: cell responses of chimpanzees immunized with irradiate sporozoites, detected by stimulating the proliferation of T lymphocytes and secretion of IFN-γ (by assay and Elispots). I.S.: Stimulation index; UI: International Units; LC: Leukocytes (mononuclear peripheral blood cells). His_6-729, PC-pGEX: recombinants belonging to the LSA3 protein; pGEX: GST protein. Threshold values are indicated by a horizontal line on the graph.

Figures 3.7(a) and **3.7(b)** illustrate by means of graphs the distribution of IgG isotypes in humoral responses against His_6-747 and His_6-772 from individuals differentially exposed to malaria. ISS: Volunteers immunized with irradiated sporozoites; SHI: Hyper-immune serum; Transfusion: Serum from persons who had contracted malaria by transfusion of infected blood. The level of responses detected by ELISA are shown with respect to the level of total IgG obtained. The standard deviation is shown on the graph.

Figures 3.8(a) and **3.8(b)** illustrate by means of graphs humoral responses for mice immunized with four recombinant protein formulations. Fig. 3.8a: anti-747 responses; Fig. 3.8b: anti-772 responses; SB: with adjuvant SBS2A; micro: recombinant adsorbed onto microparticles; IFA-incomplete Freund's adjuvant; Vi: in the form of DNA in the vector VR1020 in PBS.

DETAILED DESCRIPTION OF THE INVENTION

The originality of the present invention is based on the development of novel polynucleotide and polypeptide molecules specific to the pre-erythrocytic stage of malaria and to their uses as an active principle in an anti-malaria vaccine or in methods for diagnosing the disease.

More particularly, the invention relates to polynucleotides with a nucleotide sequence of at least 10, 20, 30, 40, 50, 75, 100, 150 or 200 consecutive nucleotides and having at least 60%, 65%, 70%, 75% and preferably 80%, 85%, 90%, more preferably at least 95%, 97% or even 100% identity with SEQ ID NO:1 or 2. Other molecules of the invention hybridize under highly stringent conditions with the above nucleotide sequences, and more particularly with SEQ ID NOs: 1 and/or NO 2. A non-limiting example of highly stringent conditions is described in the following method:

a) pre-hybridization and hybridization at 68°C in a solution containing: 5X SSPE (1X SSPE = 0.18 NaCl, 10 mM NaH_2PO_4); 5X Denhardt's solution; 0.05% (w/v) sodium dodecyl sulphate (SDS); and 100 µg/ml of salmon sperm DNA;

b) washing twice at ambient temperature for 10 min in the presence of 2X SSPE and 0.1% SDS;

c) washing at 60°C for 15 min in the presence of 1X SSPE and 0.1% SDS; and

d) washing at 60°C for 15 min in the presence of 0.1X SSPE and 0.1% SDS.

The invention also relates to polypeptides (and fragments thereof) which are derived from the above nucleotide sequences and preferably polypeptides with at least 10, 20, 30, 40, 50, 75, 100, 150 or 200 consecutive amino acids and having at least 60%, 70%, 80%, 85% and preferably at least 90%, 95%, 97% or even 100% homology with one of the sequences selected from the group formed by SEQ ID NOs: 3 to 8, 10 and 12. Other molecules of the invention contain at least 10, 20, 30, 40, 50, 75, 100, 150 or even 200 consecutive amino acids having at least 60%, 70%, 80%, 85% and preferably at least 90%, 95%, 97% or even 100% identity with SEQ ID NOs: 3 to 8, 10 and 12.

It is well known in the field how homology and identity percentages between different sequences are determined. As an example, one method for analyzing the alignment of the nucleotide and peptide sequences of the invention is advantageously the GAP GCG™ (Genetic Computer Group) program from the UNIX™ (Wisconsin Sequence Analysis Package™) suite,

the Needleman and Wunsch algorithm. The parameters used are the default parameters or the following parameters: to compare the nucleotide sequences: "gap penalty" = 50; "gap extension penalty" = 3; and to compare amino acid sequences: "gap penalty" = 5; "gap extension penalty" = 0.30.

The peptides of the present invention can be prepared using any suitable method. In particular, they can be obtained by chemical synthesis, but it is also possible to obtain them biologically using different vectors in suitable appropriated cell cultures such as that described below.

The molecules of the invention can be used as they are or they can be modified (chemical conjugates, fusion protein) if necessary. For example, it may be possible to envisage modifications (chemical or nucleotidic or peptidic) allowing the nucleotides/peptides to pass through certain biological barriers, to solubilize better, or to facilitate their incorporation into particular galenical forms, such as for example liposomes or microparticles. It should also be noted in this regard that the peptides of the present invention can be in the deglycosylated or glycosylated form, if necessary. A person who is conversant with the field of the invention could obtain different polynucleotides/polypeptides and would also be able to determine which of the polynucleotides/polypeptides obtained had a suitable biological activity.

Thus, the invention also pertains to a method for preparing a peptide of the invention, by transforming a host cell using an expression vector (plasmid, cosmid, virus, etc) comprising DNA sequences coding for the peptides of the invention, followed by culturing the transformed host cell and recovering the peptide in the culture medium.

The invention thus also concerns any vector (cloning and/or expression) and any host cell (prokaryotic or eukaryotic) transformed by said vector and comprising regulating elements allowing expression of the nucleotide sequence coding for a peptide of the invention.

More particularly, the invention relates to cells of recombinant *E. coli* containing an insert corresponding to the polynucleotides defined by SEQ ID NOs: 1 and 2. More preferably,

the *E. coli* cells are those deposited at the CNCM on 23rd May 2001 with accession numbers I-2671 and I-2672. Briefly, said cells were obtained by transforming a plasmid containing either an insert corresponding to the polynucleotides defined by SEQ ID NO: 1, or an insert corresponding to the polynucleotides defined by SEQ ID NO: 2 in the *E. coli* Dh5α strain. Each plasmid was obtained from a recombinant λgt11 phage containing the insert. PCR was carried out with primers flanking the insert and that amplified insert was digested with EcoR1 and sub-cloned into the pTreHis$_6$ vector (Invitrogen) at the EcoR1 sites.

The use of vectors for the expression of proteins and peptides in the cells of a host, in particular the human, is known and will not be described in further detail. It may be advantageous to use vectors incorporating sequences that are capable of increasing the immunogenicity of the polynucleotides/polypeptides of the present invention, such as CPG sequences, the GMCSF (granulocyte macrophage colony stimulating factor) gene, or cytokine genes. The specific constructions clearly depend on the host, the epitope and on the vector employed.

The peptides of the present invention and the polynucleotides coding for them can also be used to prepare polyclonal or monoclonal antibodies that are capable of binding (preferably specifically) to at least one peptide/polynucleotide of the invention. The present invention thus also relates to such purified antibodies which can be obtained by very well known techniques, such as the technique described by Kolher and Milstein (Continuous cultures of fused cells secreting antibody of predefined specificity, Nature (1975), 262: 495-497).

In one advantageous implementation of the invention, at least one portion of the immunogenic peptides/polynucleotides of the invention is conjugated to a support onto which it is absorbed or bound in a covalent or non-covalent manner to its C- and/or N-terminal end. The support can be constituted by carrier molecules (natural or synthetic), which are physiologically acceptable and non toxic. Said carrier molecules can increase the immunogenicity of the peptides of the invention by means of complementary reactive groups respectively carried by the

carrier molecule and the peptide. Examples of carrier molecules which can be mentioned are natural proteins such as tetanus anatoxin, ovalbumin, serum albumin, hemocyamines, PPD (purified protein derivative) of tuberculin, etc. Examples of synthetic macromolecular supports that can be mentioned for example, are polylysins or poly(D-L-alanine)-poly(L-lysine). Hydrocarbon or lipid supports that can be mentioned are saturated or unsaturated fatty acids. The support can also take the form of liposomes, particles and microparticles, vesicles, latex bead microspheres, polyphosphoglycans (PGLA) or polystyrene.

The invention also concerns vaccine/therapeutic (drug) compositions comprising the peptides/polynucleotides, conjugates and/or polyclonal or monoclonal antibodies described above, and a pharmaceutically acceptable vehicle. The invention also concerns immunogenic compositions capable of inducing protection by a challenge infection with Plasmodiums, both *in vivo* and *in vitro* and, preferably, protection by a challenge infection with *Plasmodium falciparum*. Preferably, the compositions of the invention allow the production of γ-interferon by the leukocytes of subjects immunized with irradiated sporozoites and/or the production of a humoral IgG response of the IgG1, IgG2, IgG3 and/or IgG4 type.

Said compositions may be advantageous for *in vivo* administration for the treatment or prevention of malaria in the human being. Clearly, the use of compositions based on antibody generally necessitates that they are compatible with administration to the human being. It may be antibody humanized by known techniques or directly expressed *in situ* from the DNA sequence, for example using the technique described by Ren E C, "Cellular and molecular approaches to developing human monoclonal antibodies as drugs" (1991), Ann Acad Med Singapore, 20: 66-70.

The compositions of the present invention can be in any of the usual solid or liquid forms for pharmaceutical administration, i.e., for example in liquid administration forms, as a gel, or any other support allowing controlled release, for example. Among usable compositions that can

be cited are injectable compositions, more particularly intended for injection into the blood circulation in the human being.

The compositions of the invention can also comprise components that increase or susceptible to increase the immunogenicity of peptides, in particular other immunogenic peptides, immunity adjuvants which may or may not be specific, such as alum, QS21, Freund's adjuvant, SBA_2 adjuvant, montanide, polysaccharides or equivalent compounds.

The present invention also concerns compositions intended for administration to express the peptides described above *in situ*. As an example, when injecting "naked DNA" coding for the immunogenic peptides of the invention, this injection in some cases results in expression of the coded peptide and to an immune response against said peptide. It is also possible to use naked DNA systems, but comprising their own expression system or expression vectors as described above. The expression vectors are in some cases susceptible of improving the activity of the expressed peptides. Vaccination systems employing DNA sequences are known and have already been widely described in the literature. Examples of vaccination employing DNA sequences have been described in International patent application WO 95/111307 and in the publication of Bot et al (DNA immunization of newborn mice with a plasmid expressing nucleoprotein of *influenza* virus (1996), Viral Immunol, 9:207-210).

The invention also concerns *in vitro* methods for diagnosing malaria in an individual susceptible of being infected with *Plasmodium falciparum*.

In one embodiment of the invention, the method comprises the following steps:

a) bringing a biological tissue and/or fluid removed from an individual who is susceptible of being infected with *Plasmodium falciparum* under conditions allowing an immunological reaction into contact with an antibody as defined above to allow the formation of immune complexes; and

b) detecting immune complexes formed *in vitro*.

In one implementation of the invention, the diagnostic method comprises the following steps:

a) bringing a biological tissue and/or fluid removed from an individual susceptible of being infected with *Plasmodium falciparum* under conditions allowing an immunological reaction into contact with polynucleotide/polypeptide molecules as defined above to allow the formation of immune complexes involving at least one of said molecules and antibodies that may be present in said biological tissue or fluid; and

b) detecting any immune complexes that are formed *in vitro*.

The invention also concerns kits for diagnosing malaria in an individual. In one implementation of the invention, the kit comprises the following elements:

a) at least one element selected from the group formed by: polynucleotide molecules, polypeptide molecules, and conjugates as described above;

b) reagents for constituting a medium suitable for a binding reaction between a test sample and at least one of the molecules defined in a); and

c) reagents allowing the detection of antigen-antibody complexes produced by said binding reaction, said reagents also possibly carrying a label or being susceptible of themselves being recognized by a labeled reagent.

In a further implementation of the invention, the kit comprises the following elements:

- antibodies as defined above;
- reagents for constituting a medium suitable for a binding reaction between a test sample and at least one said antibody ; and
- reagents allowing the detection of antigen-antibody complexes produced by said binding reaction, said reagents also possibly carrying a label or susceptible of being themselves recognized by a labeled reagent.

Although the description of the present invention uses the term "peptide" and "polypeptide", it is clear that the invention is not limited to compounds formed by the union of a limited number of amino acids. In fact, the flexibility of recombinant techniques enables proteins comprising a plurality of identical or different epitopes to be formed which are susceptible of improving the immunogenic activity of the final product. The present invention therefore also encompasses immunogenic polymers comprising between two and ten peptides selected from the polypeptides defined above. Similarly, the present invention includes oligonucleotides having a nucleotide sequence coding for oligonucleotides incorporating one or more polynucleotides as defined above.

The examples below illustrate other characteristics and advantages of the present invention.

EXAMPLES

The examples below serve to illustrate the scope of uses of the present invention and do not limit that scope. Modifications and variations can be made without departing from the spirit and scope of the invention. Although methods or products equivalent to those described below can be employed to test or implement the present invention, preferred materials and methods have been described.

1) INTRODUCTION

1.1 History of malaria

Malaria is a disease caused by infection of protozoic parasites belonging to apicomplexes of the species *Plasmodium* and transmitted by female mosquitoes of the genus *Anopheles*. Sustained effort and the eradication program begun in the 50s, financed by the WHO, have limited the zones in which the disease is propagated and reduced the number of infected persons. Since then, a reduction in the effectiveness of means for combating the parasite has caused an increase in cases of malaria compared with 20 years ago. Today, malaria is concentrated in the sub-tropical belt where between 300 and 500 million clinical cases are recorded annually, with a

minimum of 3 million succumbing, mainly because of infection by *Plasmodium falciparum*. Following the appearance and extension of global resistance to the only effective drugs, and because the regions affected are extending, since 1998, the WHO has classified malaria among the three infectious diseases of major interest to the world public health, alongside tuberculosis and AIDS.

A description of malarial infection, the clinical signs of which are highly characteristic, can be found in the writings of the oldest civilizations, such as the *Nei Ching*, the great medical directory of the Chinese emperor Huang Ti (2700 BC), Mesopotamian tablets (2000 BC), Egyptian papyruses (1500 BC) and the Vedic writings (1500-800 BC). Part of Hippocrates' "book of epidemics" (460-370 BC) was devoted to the detailed description of tertiary or quaternary fevers and also mentions a relationship between splenomegaly and proximity to marshy zones. The term "paludism" designates a fever deriving from marshy zones (Latin: palude = marsh), which is also reflected in the term malaria (L: mall'aria) probably introduced by Sansovino in 1560 to describe the "bad air" issuing from the marshes. Draining those zones was one of the only known means of controlling malaria prior to the discovery of the infectious agent. Despite knowing the clinical signs, the parasite causing the disease was only discovered at the end of the 19th century.

In 1880, Charles Louis Alphonse Laveran observed the exflagellation of microgametes and altered hematia in blood (Laveran, 1880) and he associated these forms with the disease. His conclusions were controversial and were only accepted by 5 years later by others, in particular by the important Italian school. The mode of transmission of the disease remained unknown for 12 more years. In 1877, Patrick Manson demonstrated that the filariosis nematode (Elephantiasis) was transmitted by a mosquito. He was convinced that malaria followed a similar path. He advised Ronald Ross to focus his research on that matter and in 1897 this one described, for the first time, oocysts in mosquitoes that had fed on infected humans. Then, using bird *Plasmodium*, he was able to describe the entire life cycle of the parasite in the mosquito.

This cycle was confirmed in 1898 for the plasmodial species in man by Italian researchers led by Battista Grassi.

For a long time it was believed that after inoculation by the mosquito, the sporozoite invaded the red blood cell of the host mammal directly, initiating the asexual and sexual blood cycle. An exo-erythrocytic cycle was described in 1908 in bird plasmodia by H de Beaupaire Aragao who demonstrated the development of atypical forms, in endothelial cells and macrophages, capable of releasing forms invading the red blood cells and of transforming into the typical pigmented forms of the parasite. However, it was believed that the tissue cycle was a particular form of those plasmodial species. It was only when observations during the course of induced malarial infections and closely followed in individuals (such as malariotherapy in the 1920-50s) were made that the presence of a supplementary tissue stage was postulated then actively researched. The pre-erythrocytic forms of the parasites of primates and humans were only discovered in 1948 when H E Shortt and P C C Garnham described hepatic forms derived from inoculations of sporozoites of *P. cynomolgi* (close to *P. vivax*) in the rhesus monkey (Shortt and Garnham, 1948). In 1951, the same stages were described for *Plasmodium falciparum* (Shortt et al, 1951) in a remarkable experiment in which a liver biopsy was removed from a volunteer who had been inoculated with millions of sporozoites. However, fresh outbreaks due to *P vivax* or *P ovale* were not explained, and the hypothesis of a "secondary" exo-erythrocytic cycle was expressed. Much after, this phenomenon was demonstrated experimentally. Then, a "dormant" stage of the hepatic form, the hypnozoite, was described in 1980 (Krotoski et al, 1980) for *P. cynomolgi*, the equivalent for the primate of *P. vivax*. This form is responsible for relapses after the parasite has been absent for a long time in the blood/exposure to parasites, and are characteristic of *P. vivax* and *P. ovale*. Recently, a supplemental stage, the merophore, a form deriving from blood forms, has been observed in the spleen and lymphatic ganglia of mice infected with murine *Plasmodium* (*P. yoelii, P. chabaudi* and *P. vinckei*) (Landau et al, 1999). This step of the cycle still remains to be described in plasmodial human species.

The parasite cycle, as understood today, is shown in Figure 1 (the portions between parentheses are forms described for other species of *Plasmodium*, but not for *Plasmodium falciparum*).

1.2 Means for combating the spread of malaria: the need for developing a vaccine

Since the start of the twentieth century, the two discoveries of the causal agent of the disease and of the disease vector have allowed rational defenses against malaria to be developed against malaria by attacking the parasite in the vertebrate host using drugs or by targeting the mosquito vector either with larvicides, or with insecticides, or using mosquito nets. The success in eliminating the disease in temperate zones after the second world war has led to the development of a malaria eradication program which culminated in the 60s when DDT was the principal tool against mosquitoes and chloroquine was the principal drug against the parasite. The size of the targeted endemic territories was thus reduced (quasi eradication in temperate zones) and the number of persons affected by the disease was initially reduced. However, the successes in tropical zones were short-lived. The number of patients has not stopped increasing, partly because of demographic increases, partly because of the appearance of resistance to insecticides and to the available drugs.

The appearance of resistance has required re-orientation towards other combating means. The existence of a natural immunity induced by exposure to parasites and the observation that the passive transfer of immunoglobulins from immune persons reduces parasitemia, and effective and sterilizing immunization by sporozoites attenuated by irradiation have rendered reasonable the postulation of a vaccine against malaria, the development of which thus constitutes a public health priority on a global scale.

1.3 The search for salvation by immunity

Natural immunity against malaria is characterized by very slow development and the fact that it does not result in sterilizing protection. In hyperendemic zones, the acquisition of natural immunity against the erythrocytic stages manifests itself in children initially by tolerance to the

parasite (anti-toxic immunity) then with age by a reduction in the parasite load in the blood (anti-parasitic immunity).

These observations, made during epidemiological studies, were confirmed by experimental infections. Malaria therapy applied to persons with neurosyphilis (Boyd and Coggeshall, 1938; Ciuca et al, 1943; James, 1936), allowed parameters to be defined which were involved in the acquisition of immunity in carefully controlled experiments. It was shown that acquired immunity was firstly dependant on the species and on the strain and secondly differed as a function of the stage of the infecting parasitic cycle. Until now, the precise mechanisms of anti-malarial immunity remain to be elucidated.

Because the clinical signs and transmission are uniquely due to the blood stages and that these are the most accessible both *in vitro* and *in vivo*, the majority of vaccine studies have concerned these stages. About thirty antigens expressed in erythrocytic parasites have been identified, particularly by monoclonal antibodies, and considered as vaccine candidates. However, tests with inducing protective immunity by the rare antigens that have been tested in man have remained fruitless until now.

The first immunization with pre-erythrocytic stages was attempted by the Sergent brothers in Algeria (Sergent and Sergent, 1910). The capacity of protecting in a sterilizing manner (absence of any blood parasitemia) was only obtained by immunization with sporozoites attenuated by irradiation. This approach was initiated by studies in the bird with sporozoites irradiated with UV radiation (Mulligan et al, 1941), and were repeated 20 years later with rodent plasmodia using sporozoites irradiated with X rays and later with γ rays, the dose of which could readily be controlled (Nussenzweig et al, 1967; Richards, 1966); immunity could be maintained by repeating with non-attenuated sporozoites (Orjih et al, 1982). In man, such protection was also obtained (Clyde, 1975; McCarthy and Clyde, 1977); however it was only induced after a very large number of inoculations with irradiated sporozoites and so such a vaccine procedure cannot be applied on a large scale.

For a long time, it was believed that protection was correlated with an observed phenomenon when sporozoites were incubated with immune serum, CS (Circum sporozoite) precipitation (Vanderberg et al, 1969). The major protein recognized by that serum, the CS protein, was thus considered to be responsible for that immunity. Since then it has formed the basis of many vaccine studies in many experimental models. However, until now, none of the studies has been able to reproduce an immunity as good as that induced by irradiated sporozoites.

A critical evaluation of the previous experimental results has led to the postulation that the hepatic stage and not the sporozoite is at the origin of sterilizing immunity (Druilhe and Marchand, 1989). The principal indication was the fact that protection could only be induced by inoculation with viable sporozoites, intravenously, capable of invading a hepatocyte and developing therein, and that hepatic forms derived from irradiated sporozoites persisted (Ramsey et al, 1982). Further, eliminating the hepatic stages would cause susceptibility to infections by sporozoites in previously protected animals (Londono et al, 1991; Scheller and Azad, 1995).

The hepatic stage has unique characteristics. The hepatocyte is a nucleated cell that is metabolically highly active and expresses molecules of the major histocompatibility complex. Hepatic schizogony causes the formation of between 10000 and 30000 merozoites while 4 to 32 merozoites are released by a blood schizont. Merozoites from these two stages have morphological differences, but it is not known whether functional or molecular differences exist as only blood merozoites have been able to be studied extensively.

Because only a few hepatocytes in the liver are infected and that *in vitro*, culture techniques remain delicate and difficult, this has constituted a major obstacle to developing knowledge regarding the hepatic stage and the search for antigens expressed at that stage.

1.4 Screening for specific stage antigens

The first strategy for establishing stage-specific expression is the generation of libraries of complementary DNA from messenger RNA from different stages. This was accomplished

several times for the blood stages (Chakrabarti et al, 1994; Watanabe et al, 2001) and more recently once for the sporozoite stage (Fidock et al, 2000). However, that approach is not possible for the hepatic stage of human plasmodia. A further mean is the generation of specific antibodies in animal models. This is easy for the erythrocytic stages but for the hepatic stage, a number of attempts have failed as injecting the hepatic stages of *Plasmodium falciparum* have only induced a very few antibodies in mice. A final approach is immunological screening based on the use of antibodies from naturally immunized individuals. That approach has demonstrated, for the first time, that antigens other than CS are present on the sporozoite surface (Galey et al, 1990).

1.5 Laboratory strategy for identifying antigens expressed at the pre-erythrocytic stages

In order to overcome the difficulty of screening at pre-erythrocytic stages, a strategy for screening *Plasmodium falciparum* antigens potentially expressed in the sporozoite and hepatic stages has been developed (Marchand and Druilhe, 1990).

The principle was to seek individuals in whom the predominant immune response was against the pre-erythrocytic stages. We obtained serum from individuals (PM serums) living in an endemic zone for over 20 years and who had never clinical events as they were permanently under prophylactic treatment with chloroquine (a schizonticide effective against the blood stages, but with no effect on the hepatic stages). The corresponding serum only weakly recognized blood stages under Western Blot and IFI (titers of less than 1/200), while titers against the sporozoite and hepatic stages of the parasite were in the range 1/3200 to 1/6400 in IFI and they labeled several polypeptides on protein extracts from *Plasmodium falciparum* sporozoites; the serum thus contained antibodies specific to antigens expressed in the pre-erythrocytic stages.

Those serums were used to screen a gene library from *Plasmodium falciparum* (constructed by Odile Mercereau Puijalon). The genomic DNA from the parasitic clone T9-96 was methylated and digested with Dnase 1, and fragments with a size of 200 to 2500 base pairs were introduced into the EcoR1 site of the λgt11 phage (Guérin-Marchand et al, 1987).

Of the 7 million fragments of DNA that were generated, 2000 clones producing a recombinant antigen recognized by hyperimmune serum (HIS) from immune individuals living in the endemic zone were then screened with the PM serum. 120 clones were then selected and stage-specific expression of the corresponding antigens was determined by IFI tests, with immunopurified human antibodies on each recombinant protein, on sporozoites, the hepatic stages and the blood stages of *P. falciparum*, *P. yeolii* and occasionally with *P. berghei* and *P. vivax*.

The first antigen to be studied and against which the humoral responses were the greatest in several serums from individuals living in an endemic zone was the Liver Stage Antigen, LSA-1 (Guérin-Marchand et al, 1987). It remains the only characterized antigen to be expressed uniquely at the hepatic stage.

Following LSA-1, 3 antigens, STARP, SALSA and LSA-3, were selected from the various criteria and characterized on the molecular level (Bottius et al, 1996; Daubersies et al, 2000; Fidock et al, 1994), and immunologically by L. Benmohammed, K. Brahimi, J.-P. Sauzet and B Perlaza (BenMohammed et al, 1997; Perlaza et al, 1998; Sauzet et al, 2001). Those antigens are expressed both on the sporozoite surface and in the hepatic stage.

LSA-3 is the only antigen that is differentially recognized by serum from volunteers or chimpanzees protected by immunization with irradiated sporozoites. It is the only one to have induced sterilizing and long term protection in chimpanzees (Daubersies et al, 2000), and will soon be tested in phase I and II clinical trials.

2) MATERIAL AND METHODS

2.1 Molecular biological techniques

2.1.1. Bacterial strains

DH5α: *supE*44 Δ*lac*U169(φ80 *lacZ* ΔM15) *hsdR*17 *rec*A1 *gyrA*96 *thi-* 1 *relA*1.

2.1.2. Parasite strains:

NF54 from an isolate from a European patient infected in Africa (ATCC MRA151) (Walliker et al, 1987).

3D7, the reference strain used in the genome project, is a clone from the (ATCC MRA151) strain (Walliker et al, 1987).

T9.96, a strain from a Thai patient, ATCC: MRA153, (Thaithong et al, 1984).

For the polymorphism tests, distinct strains were employed: B1 (Brazil); F32, D7, D28, D50 from Tanzania; D28 from Senegal, D41 from India; D51 from Myanmar, L1 from Liberia; H1 from Honduras, Mad20 from Papua New Guinea, and PA from Palo Alto, South West America (Stricker et al, 2000).

The sporozoites were derived from the NF54 strain and obtained by passage through *Anopheles Gambiae* REF.

2.1.3. PCR from phage extracts or phage DNA

The Expand High Fidelity Kit™ (Mannheim Boehringer, Germany) was used as indicated by the supplier with 2 mM of $MgCl_2$, 3.5 units of Taq polymerase, 0.2 mM of deoxyribonucleotides (dNTP), 50 nM of 21D primers 5' (CCTGGAGCCCGTCAGTATCGGCGG; SEQ ID NO: 13) and 26D primers 3' (GGTAGCGACCGGCGCTCAGCTGG; SEQ ID NO: 14) and 2 µl of purified DNA or phage extract. The reaction comprised initial denaturation for 2 minutes at 94°C, followed by 35 consecutive cycles of 15 seconds of denaturation at 94°C, 30 seconds hybridization at 50°C, and 2 minutes elongation at 68°C. The cycle was followed by incubation at 68°C for 5 minutes.

2.1.3.1. Sub-cloning in histidine, pNAK and Topo vectors

Depending on the amplification product of the phages, three procedures were employed:

The PCR products with a smear or a very small yield and being smaller and almost impossible to detect by digesting the DNA of the corresponding phage were cloned using a vector allowing direct cloning of the PCR product without successive digestion of a restriction

enzyme using the TopoTA Cloning™ kit (Invitrogen, Netherlands). Topo cloning was also carried out for fragments for which only the sequence was to be determined.

PCR products with a size of less than 1 Kbp were digested, precipitated with ethanol and re-suspended in half of the initial volume of H_2O, then digested with 10 U of the restriction enzyme *Eco*R1 for 1 hour at 37°C, separated on a 2% agarose gel, purified on gel using the Qiagen gel extraction kit to give a volume of 50 µl.

Large less abundant PCR products (more than 1000 bp) were isolated from phage DNA purified by digestion with EcoR1, then by extraction of the insert on agarose gel.

2.1.3.2. Study of gene polymorphism in different parasitic strains

The following primers were used to identify size polymorphisms of specific regions corresponding to the antigens studied.

747-1: AAAAGTGATGATAGAAATGCTTGTG (5'); SEQ ID NO: 15

747-2: TTTTGTTGATCTTACTTATTTCACC (3'); SEQ ID NO: 16

772-1: CGGAATCAGGTTTAAATCCAAC (5'); SEQ ID NO: 17

772-2: AGATCGTTTTTCATCAGGGGG (3'); SEQ ID NO: 18.

The cyclic reaction was carried out using a program comprising an initial denaturation step at 94°C for 15 seconds, followed by 39 cycles comprising denaturation at 94°C for 2 minutes, hybridization at 52°C for 1 minute and elongation at 72°C for 2 minutes. A 5 minute step at 72°C terminated the reaction.

PCR was carried out using an Appligène Crocodile III™. The products were then analyzed on agarose gel.

2.1.4. DNA purification

2.1.4.1. Recombinant analysis

The positive PCR colonies were inoculated into 3 ml of medium containing the antibiotic corresponding to the vector used (100 µg/ml of ampicillin for Topo and Histidine, 20 µg/ml of kanamycin for the Vical vector) and 2 ml of the inoculum was used in preparing the plasmidic

DNA with the Qiagen™ Miniprep Kit. The DNA obtained was successively digested with the restriction enzymes used in cloning and underwent to an agarose gel electrophoresis, to detect insertion of the fragment.

2.1.4.2. Fragment isolation

100 ml of Luria Broth medium supplemented with a suitable antibiotic was inoculated with 1 bacterial colony comprising the recombinant and incubated at 37°C overnight in a thermostated bath with vigorous agitation. The next day, the bacterial culture was harvested and the plasmic DNA was purified as described (Qiagen maxiprep™, Qiagen, Germany).

2.1.4.3. Immunization of naked DNA constructs

In order to eliminate endotoxins, which are present in bacteria and which can cause non specific responses during mouse immunizations, the DNA of the constructs was purified from 2 l of recombinant bacterial cultures, using the Qiagen EndoFree Plasmid Giga™ kit (Qiagen, Germany).

2.1.4.4. Purification of recombinant DNA phages

The phages were re-amplified on LB agarose dishes, by depositing 5 µl onto Topagar taken with 200 µl of Y1090 inoculum and leaving at 37°C overnight.

A larger quantity was then produced in liquid culture. Firstly, a plaque pricked onto the dish was incubated with 200 µl of Y1090 inoculum and left at 37°C with stirring for 15 minutes. Then 5 ml of antibiotic-free medium supplemented with 10 mM of $MgSO_4$ was added, and the culture was left with stirring for 4 hours until lysis occurred. 50 µl of Chloroform was added and it was centrifuged at 7000 g for 10 minutes. After centrifugation, the supernatant free of cell debris was recovered. This stock was used to produce 500 ml of liquid culture phage: the equivalent of 7.5×10^8 pfu (plaque-forming units) was added to 500 µl of cells of a culture inoculated overnight with Y1090, and 500 µl of 10 mM $MgCl_2/CaCl_2$. It was incubated at 37°C for 15 minutes and added to 500 ml of antibiotic-free LB medium. Lysis of the bacteria observed by the appearance of filaments in the culture was followed until lysis was complete (4-

5 h). Then the culture was centrifuged at 6000 g for 15 minutes at 4°C, the supernatant was recovered and stored at 4°C overnight.

The next day, the DNA was purified with the Lambda Maxi Kit™ (Qiagen, Germany) adjusting the start of the protocol with a larger volume of starting supernatant. The final residue was re-suspended in 500 μl of TE buffer.

2.1.4.5. From parasites

100 μl of culture residue from red blood cells with 10% parasitemia was re-suspended in 100 μl of PBS, pH 7.2 and purified using the Qiaamp DNA Mini Kit™ (Qiagen, Germany). About 5 μg of DNA was obtained from 100 μl of the residue of the 10% parasitic culture.

2.1.5. Purification of total parasitic RNA

We used two methods, depending on the desired quantity of RNA. For large quantities, the method described by Kyes et al (2000) was used, while to obtain preparations in more restricted quantities, we used the RNeasy Kit™ (Qiagen, Germany).

2.1.6. RT-PCR

RT-PCR was carried out using the RT-PCR kit of Qiagen (Germany). Specific primers for each gene and situated, if possible, so that it was possible to distinguish between the products from amplification of genomic DNA and RNA (around the introns) were used. A first reverse transcription reaction was carried out at 50°C for 30 minutes, then a PCR reaction was carried out under the same conditions as those described for PCR of parasitic DNA with selected primers, sometimes followed by a second reaction (nested PCR) with primers located in the sequence for the first amplified PCR product. However, the hybridization temperature varied as a function of the primers used (between 50°C and 60°C).

2.1.7. Purification of histidine recombinants

2 l of Luria Broth medium supplemented with 100 ng/ml of ampicillin was inoculated with 50 ml of bacterial culture containing the recombinant plasmid. The growth of bacteria was followed by measuring the bacterial turbidity at 600 nm and at the desired optical density

(between 0.5 and 1), a concentration of IPTG in the range 0.5 and 1 mM depending on the recombinant was added to the culture and induction lasted between 2 h and 4 h.

The cells were then harvested and the bacterial residue was re-suspended in a buffer of 20 mM of $NaPO_4$, pH 7.4 and 8 M of urea (TU) (25 ml/liter of bacterial culture). The cell suspension then underwent sonication, 10 shocks of 1 minute each, and the supernatant containing the recombinant proteins was recovered by centrifugation at 10000 g for 10 minutes, and filtered at 0.22 µm. An affinity purification step was carried out on a Nickel column. A 1 ml column (HiTrap™, Pharmacia, Sweden) was washed as indicated by the supplier and 1 ml of $NiCl_2$ was applied, followed by others washes. The column was then washed with 5 ml of TU, and the supernatant was applied to the column. A wash with 10 ml of TU was then carried out ant the recombinant eluted with an increasing gradient of imidazole, a competitor for histidine. Depending on the purified recombinant, different concentrations were used, and the results obtained are summarized in the table below. The protein pool was then dialyzed against a pH 6 L-histidine buffer, and chromatographed on an anion exchange column (HiTrapQ™, Pharmacia, Sweden) to eliminate a portion of the Lipo Poly Saccharides (LPS) or endoxins which induce non-specific responses (Morrison and Ryan, 1987).

Histidine recombinant purification table

Recombinant	Induction[1]			OD after induction	Protein location, molecular weight[2]	Imidazole (mM)[3]	NaCl (mM)[4]
	OD	IPTG	Time				
747	0.5	0.5	4h	2.7	Mem, 18	50	360
772	0.5	0.5	4h	2.2	SN, 35	36	120

1: OD measured at 600 nm, the concentration of IPTG (mM); and the induction time before harvest;

2: After sonication, and centrifugation of a suspension of bacteria containing no urea (8M) in the buffer, the supernatant and the residue containing the bacterial membrane debris was tested using Western blot to detect where the recombinant protein was located. In the presence of urea all proteins were soluble and the purification procedures were thus applied in the presence of 8M urea.

3: The concentration of imidazole at which the protein was eluted on the HiTrap-Ni™ column.

4. The concentration of NaCl at which the protein was eluted on the HiTrap-Q™ column.

2.2. Immunological techniques

2.2.1. ELISAs (Enzyme Linked ImmunoSorbant Assay)

The optimum conditions were determined with 100 μl of antigen solution at a concentration of 10, 5 or 1 μg/ml coated onto plates in 50 mM of Carbonate, pH 9.6 or 1X PBS, pH 7.4 by incubating plates overnight at 4°C. Saturation was achieved, either in PBS supplemented with 3% of skimmed milk, or 1% of BSA (calf serum albumin) at ambient temperature or at 37°C for 2 hours. Dilution of serums 100 or 200 times was carried out either with 1.5%PBS/milk, or with 1%PBS/BSA, and incubation was carried out at ambient temperature or at 37°C for 1 h.

Incubation with secondary antibodies coupled with HRPO (horseradish peroxidase) diluted by 1/2000 in the serum diluting buffer was carried out at ambient temperature, and visualizations were done using TMB buffers (peroxidase substrate and peroxidase solution B) (Kirkegaard and Perry Laboratories, USA) mixed volume for volume immediately prior to use, 100 μl of which was distributed in each well. The blue stained reactions were stopped by adding the same volume of a 1 M solution of phosphoric acid. The reactions were viewed at 450 nm in a Multiscan Ascent™ (Labsystems) reader.

The results with mice are expressed as a Ratio (an arbitrary unit with respect to the level of response in naïve controls) and in the experiments in which the number of isotypes were studied, as the ratio of total IgG determined in the same experiment.

2.2.2. Immunopurification of specific antibodies

For the immunopurification of specific antibodies against His_6 recombinants, a method described by Brahimi et al, (1993) was employed. 100 μl/well of the antigen solution in PBS, pH 7.2, at a concentration of 5 μg/ml was adsorbed onto Nunc Maxisorp™ plates (Nunc, Denmark), and the plates were incubated at +4°C overnight. The hyperimmune serum was then incubated at a dilution of 1/50 for 1 hour at ambient temperature, the plates were washed and the antibodies were eluted by adding glycine at 0.2 M pH 2.5, , incubation for 3 minutes and

recovering followed by neutralizing the pH with Tris, 1M, pH 11. Immunopurification from β-galactosidase fusion recombinants was carried out on nitrocellulose filters, as described by Beall and Mitchell, (1986).

2.2.3. SDS-PAGE and Western Blot

Depending on the test samples, gels with different percentages of acrylamide (BioRad™ 29.1:1 ratio) (5, 7.5, 10 or 12%) were used. After migration in a Tris/glycine buffer (pH 8.5) with the minigel kit (Biorad, USA), the gels were either stained with Coomassie blue or underwent transfer to nitrocellulose filters (0.45 μm) in the Trans-Blot™ cell (BioRad).

After transfer, the proteins were viewed by staining with 0.2% of Ponceau red in a solution of acetic acid (5%), then the filter was saturated with TBS/5% skimmed milk for 30 minutes. The human antibodies, immunopurified without dilution, and the serum diluted to 1/100 or 1/200 in TBS/5% milk/0.05%Tween™, were incubated for 1 to 2 hours at ambient temperature. The filter was then washed 3 times for 10 minutes in TBS/0.05% Tween™ and incubated with antiserums coupled with alkaline phosphatase diluted to 1/5000 for 30 minutes. After washing in the same buffer, color reactions were produced by adding NBT (330 μg/ml) and BCIP (165 μg/ml) (Promega, Germany) diluted in Tris buffer, pH 9.

2.2.4. IFI (Indirect ImmunoFluorescence)

All incubations at 37°C were carried out in a moist chamber to avoid drying out the tissues or cells to be studied. The buffers were filtered with a 0.22 μm filter to prevent contamination by other microorganisms and background noise.

<u>2.2.4.1. Sporozoite stage</u>

After dissection of the salivary glands of mosquitoes infected with the parasite, the sporozoites were fixed with 0.01% of glutaraldehyde in PBS and washed carefully with PBS.

In order to study labeling only on the sporozoite surface, Galey et al, (1990) developed a technique for "wet" fixation with a suspension of sporozoites attached to polylysin. The titration slides (Polylabo, France) were coated with 1 μl of 50 mg/ml polylysin solution then left to dry

overnight at 37°C. 1 μl of a suspension of sporozoites (20/μl) was deposited on each well and incubated overnight in a moist chamber at 4°C. Intra-parasitic detection was carried out by fixation of the sporozoites in acetone.

2.2.4.2. Hepatic stage

Sections fixed with Carnoy's fixative and paraffined were prepared by 3 baths of xylene each for 10 minutes, 3 baths of absolute alcohol, each of 5 min, 2 baths of distilled water, each of 5 minutes, and dried in the open air. The sections were then rehydrated for 10 minutes in filtered PBS, pH 7.4. Sections for freezing were fixed in acetone for 10 minutes.

2.2.4.3. Blood stage

Blood slides were fixed in acetone for 10 minutes and compartments for each test sample were defined by drawing edges with a Pentel red label on the smear.

The remainder of the technique was identical for each of the three stages: after fixing, the test antibodies (diluted in PBS) were deposited into each well, cup or compartment, and the slide was incubated at 37°C in a moist chamber for 1 hour. The slides were washed 3 times for 10 minutes in 1X PBS, then incubated with an anti-human or mouse anti-IgG (depending on the specific antibodies used), coupled with fluorescein (Alexis) diluted by 1/200 in PBS and 1/50000 Evans blue, incubated for 30 minutes at 37°C in a moist chamber, washed three times in 1X PBS, and covered with a slide after one drop of glycerin buffer (PBS, 30% glycerol) had been deposited. The slide was observed under a UV microscope (Olympus™ BH2).

2.2.5. Mouse immunizations

2.2.5.1. With recombinant histidines

Protocols a, b and c were essentially employed to obtain specific serums, while protocols b, c and d were used to carry out challenge infections with *P. yoelii*.

a) IFA/alum adjuvant

Female 6-week old BALB/c mice received a first intraperitoneal injection of 500 μl with a mixture of 20 μg of antigen (His_6-249, His_6-680, His_6-747, His_6—772), 2 mg/ml of alum

($Al(OH)_3$), and incomplete Freund's adjuvant (AIF), volume for volume, supplemented with 0.9% NaCl.

The two subsequent injections, each at fortnightly intervals, were carried out with the same quantity of antigen in the same volume, but without AIF, and with methiolate, a preservative, in an amount of 0.05%.

The mice were sampled (500 µl) 2 weeks before immunization, 1 month and 6 weeks after the first immunization, onto EDTA and the plasma was recovered and stored at -20°C.

b) CFA

Female 6 week old BALB/c mice received 3 subcutaneous injections every fortnight at the base of the tail of a mixture constituted by 100 µl of complete Freund's adjuvant and 10 µg of antigen (His_6-114 or His_6-662) in 100 µl of PBS. 1 week after the third injection, mouse serum was removed and the responses were tested using ELISA against the recombinant and using IFI on the sporozoites. 18 days after the final injection, the mice were subjected to a challenge infection with *P. yoelii* sporozoites.

c) $SBAS_2$ (Smith and Klein Beecham adjuvant)

Female 7 week old C3H mice received three subcutaneous injections at the base of the tail of 100 µl of a mixture constituted by 57 µl of adjuvant mixed with 43 µl of antigen (His_6-249, His_6-747 or His_6-772) corresponding to 10 µg, the injections being separated by 3 weeks each time. 10 days after the last immunization, the mice were sampled and the corresponding serum was harvested.

d) Microparticles

The antigen solutions (His_6-249, His_6-747 or His_6-772) was adsorbed onto polystyrene microparticles 0.5 µm in diameter (Polysciences Inc, USA) by incubation at 37°C with agitation for 4 hours in a glycine solution, pH 8.0. Adsorption of the antigen was verified by the capacity of the microbeads to agglutinate with a serum specific to the adsorbed antigen. Female 7 week old C3H mice received three subcutaneous injections at the base of the tail of 100 µl of a mixture

constituted by microbeads coated with the antigen corresponding to 10 µg, the injections being separated by 3 weeks each time. 10 days after the final immunization, the mice were sampled and the corresponding serum was harvested.

2.2.5.2. With recombinant DNA

6 week old BALB/c and C3H mice were injected three times at 8 week intervals intramuscularly with 100 µl of antigen (pNAK114, pNAK249, pNAK438, pNAK571, pNAK747, pNAK772) in PBS, pH 7.4, then a fourth time 12 weeks after the third injection. Blood was sampled 1 week after the third and fourth immunization onto EDTA, and the serum was harvested after incubation of the sample overnight at 4°C.

The spleens from 3 mice/group were removed after the fourth immunization and the cell response stimulation was studied. After a fifth booster, 8 weeks after the fourth injection, the mice underwent a challenge infection with *P. yoelii* sporozoites. .

2.2.6. Challenge infection with sporozoites and blood stage

Sporozoites from *Anopheles stephensii* mosquitoes infected with the 1.1 clone from *P. yoelii yoelii* were obtained by a method (Ozaki et al, 1984) consisting of isolating the thoracic cage of the mosquito and obtaining sporozoites by centrifugation through glass wool, which sporozoites were then washed by successive re-suspension in PBS after centrifugation.

The mice were infected with *P. yoelii* sporozoites retroorbitally with 150 to 200 sporozoites (200 µl/injection) and parasitemia was monitored by smears on day 3 following infection until the 12th post-infection day, both in immunized animals and in naïve mice infected with the same batch of sporozoites.

Blood stages removed from other mice infected with *P. yoelii* were washed with PBS and the equivalent of 5 x 10^4 parasites was injected intraperitoneally.

2.2.7. Study of cell responses

To study both the induction of specific T cells proliferation and the secretion of cytokines capable of stimulating the immune response, we studied the stimulation of mouse splenocytes by antigens and the secretion of IFN-γ by these cells.

2.2.7.1 Proliferation of T lymphocytes

The spleens were removed from mices; suspensions of splenocytes were washed twice in RPMI 1640™ (Gibco, France) and the cells were re-suspended to a final concentration of 5×10^6 cells/ml in RPMI supplemented with 100 U/ml of penicillin, 2 mM of L-glutamine, 10 mM Hepes, 50 μM β-mercaptoethanol, 1.5% of foetal calf serum (FCS) and 0.5% of normal mouse serum. 100 μl/well of each suspension was distributed into 96-well round bottom plates (Costar, USA) and the recombinant proteins to be tested were added in a concentration of 50 mg/ml. These tests were carried out in triplicate. After 48 hours of incubation (37°C with 5% CO_2), 50 μl/well of culture supernatant was removed and stored at -70°C before determining the IFN-γ titer. 50 μl/well of supernatant was removed to assay the cytokines. In order to detect DNA replication due to stimulation of division, 50 μl of a solution of tritiated thymidine (^{3}H) (Amersham Life Science, England) at 1 μCi/well was added during the last 12 hours of incubation. The cells were harvested in an automatic cell harvester (Skatron Inc, Sterling, VA, USA), and incorporation of ^{3}H Thymidine quantified by scintillation. The results were expressed as the Stimulation Index (SI) and the proliferation was considered to be positive when the S.I. was above 2.

2.2.7.2 Detection of γ interferon (IFN-γ) secretion

The titers of IFN-γ in culture supernatants were determined using a sandwich ELISA method. Maxisorp™ plates (Nunc, Denmark) with flat bottoms were coated with a rat monoclonal antibody anti-primary mouse-IFN-γ (R4-6A2) (Pharmingen, San Diego, CA) diluted in a 0.1 M carbonate buffer, pH 9.6, and left overnight at 4°C. Between each step of the procedure, the plates were washed several times with PBS buffer supplemented with 0.05% Tween™ (PBS-T). The plates were then saturated with 3% bovine serum albumin (BSA, Sigma

Chemicals, St Louis, USA) in PBS-T. Non-diluted supernatants were added to the wells and the plates were incubated overnight at 4°C, followed by incubation for 1 h at ambient temperature with a secondary biotinylated rat anti-mouse IFN-γ monoclonal antibody (XMG1.2™, Pharmingen, San Diego, CA) diluted in PBS-T. The steps for labeling with antibodies coupled with peroxidase were identical to those used in the ELISA technique (A.2.1).

2.2.7.3. Detection of cells secreting IFN-γ by Elispot

The number of cells secreting IFN-γ was determined in non stimulated splenocytes 40 hours after being freshly isolated and incubated with antigens. Microtitrating plates (Multiscreen-HA™ sterile plate, Millipore) were coated with 50 μl of a solution containing 5 μg/ml of anti-IFN-γ antibody (18181D™, Becton Dickinson Co). After incubating overnight at 4°C, the wells were washed and saturated with a 5% FCS solution. Suspensions of cells at 5×10^5 cells/well were incubated with the antigen in an amount of 50 μg/ml in a total volume of 200 μl for 40 h at 37°C in a moist atmosphere with 5% CO_2. The plates were then washed three times with PBS-T and three times with PBS alone and the wells were then coated with 50 μl of biotinylated anti-mouse IFN-γ antibody solution (Becton Dickinson Co, USA) diluted to 1/200 and incubated overnight at 4°C. The plates were then washed in the same manner as before, before adding 50 μl per well of alkaline phosphatase coupled with streptavidin (Boehringer-Mannheim, Germany) in a dilution of 1/2000 in PBS. After incubating for 1 h, and washing the plates, spots were detected by developing a colored reaction with BCIP/NBT reagents at 50 μg/ml in the region in which individual cells had secreted IFN-γ. The results are expressed as the number of cells forming spots with respect to 5×10^6 splenocytes.

2.2.8. Serums and cells

2.2.8.1. From individuals naturally exposed to the parasite

10 serums from adults living in a highly endemic zone (Ivory Coast) and naturally protected were employed in ELISA studies and immunopurifications of antibodies specific to the antigens being studied.

Serum from individuals in two age ranges of 0-9 years or over 12 years were selected from Ndiop and Dielmo villages (Rogier and Trape, 1995; Trape et al, 1994). Ndiop is located in an endemic zone which records about 20 infectious bites/year, and Dielmo, in a zone which records 150 infectious bites/year. Each serum in one of the two regions corresponded in age and sex to a serum from the other region.

2.2.8.2. From animals or humans immunized with irradiated sporozoites

Two chimpanzees were immunized either with sporozoites irradiated at 18 kRad, or at 30 kRad by 4 injections each of 5×10^6 sporozoites, intravenously. The first 3 immunizations were carried out at 1 month intervals, while the 4th was carried out 4 months after the third. Their serum and peripheral blood cells were studied in cell response tests and humoral response tests after 3 immunizations. The two animals were infected by intravenous injection of 4×10^4 sporozoites (low dose) each of *Plasmodium falciparum* and only the chimpanzee immunized with 18kRad irradiated sporozoites was protected (did not develop blood parasitemia).

Two human volunteers immunized by the same means received a booster with a new batch of irradiated sporozoites, and peripheral blood cells were studied in Elispot tests. Further, the serum from 4 human volunteers immunized with irradiated sporozoites was also at the disposal of the Applicant.

2.2.8.3. From individuals exposed differentially to the parasite

We had at our disposal serum from 8 individuals naturally exposed to the parasite but under permanent chloroquine treatment, which eliminated the blood stages at a very early form and the serum from 5 individuals accidentally infected by transfusion of blood infected with *Plasmodium falciparum*.

3) RESULTS

3.0: Example 1

Identification of two novel antigens DG47 and DG772 from *Plasmodium falciparum* recognized by volunteers immunized with irradiated sporozoites

The DG747 and DG772 clones were selected not simply because of the initial criteria imposed (detection on sporozoites and the hepatic stage, and recognition by hyperimmune serum), but because several supplementary characteristics interested us: DG747 had no cross reactivity with other proteins from the PM library, and DG772 had only one cross reactivity, with LSA-1, the only antigen identified as being expressed only at the hepatic stage of *Plasmodium falciparum*. Further, specific antibodies for the two proteins labeled *P. yoelii* sporozoites.

An initial sequencing revealed that these two clones contained inserts belonging to genes that were unknown until now, but the sequence of which was available on databases for the *Plasmodium falciparum* genome. We thus decided to work on the molecular characterization of stage expression, gene conservation, and an immunological characterization (antigenicity, immunogenicity) of these novel antigens. The results show that a) these two antigens induced an immune response in individuals or animals exposed only to the pre-erythrocytic stages both artificially (by immunization) and naturally (on the ground); b) they are recognized by serum from individuals naturally exposed to the entire life cycle of the parasite, both in zones of weak and of highly endemic nature. Further, we have evaluated in the mouse their immunogenic potential and protective potential by immunization and challenge infection by *P. yoelii*.

3.1. Sequence analysis

DG747 codes for a 59 amino acid polypeptide the 40 C-terminal amino acids (aa) of which form part of a repetitive structure of 5 x 8 aa rich in arginine and lysine. This sequence is identical to aa 81-140 of the PfB0155c gene (1524 bp, 508 aa) located on chromosome 2 (Figure 3.1a). This gene, which codes for a putative protein (Gardner et al, 1999) comprises neither the predicted introns nor signal peptides, nor regions homologous with other proteins from *Plasmodium* or other organisms. The corresponding protein has a theoretical molecular mass of 59 kDa, and a neutral isoelectric point (Ip) (7.5), but certain regions have highly variable Ip, for example the region found in DG747 has a positive charge at neutral pH.

DG772 contains a 333 bp insert, which are translated into 111 aa contained in an open reading frame. This polypeptide corresponds to the region of 1146-1256 aa of a protein with 1493 amino acids coded by a gene located on chromosome 1 (Figure 3.1b). The theoretical mass of the protein is 173 kDa and the isoelectric point is 5.05. The protein is mainly constituted by polar amino acids and does not contain hydrophobic sites, at least in the N-terminal portion, where it may have a GPI anchoring site. The gene contains no repetitions and the translated nucleotide sequence has a great homology with proteins of the "EBP" family (Adams et al, 1992), i.e. with the 5'cys and 3'cys regions which are characteristic of this family.

3.2. Stage expression and gene conservation

In order to evaluate stage expression of the two proteins more precisely, we used IFI and Western Blot techniques on different stages of *Plasmodium falciparum* and on murine parasites *P. yoelii* and *P. berghei*.

The surface of *Plasmodium falciparum* sporozoites was labeled with antibodies (human or mouse) specific to DG747 and DG772, but the erythrocytic stages were labeled differently for the two groups of antibodies. The anti-His_6-747 (anti-747) antibodies labeled the young stages but little, and labeled the mature schizont stages strongly, with localized labeling around the knob structures (Figure 3.2 image A), while the anti-His_6-772 (anti-772) antibodies labeled the parasite in a more homogeneous manner throughout the erythrocytic stage. In the murine species *P. yoelii* and *P. berghei*, the surface of the sporozoites was strongly labeled by the specific antibodies of the two antigens.

In order to define the size of the detected proteins, we also carried out Western Blot on protein extracts from blood parasites of *Plasmodium falciparum* with the same antibodies (Figures 3.3a and b).

The anti-747 antibodies labeled a polypeptide of about 70 kDa both in ring extracts and in schizont extracts, while no band was detected in non parasitic erythrocytes. The polypeptide detected by anti-772 antibodies was larger, with a molecular mass of 150 kDa, and was detected

both in the rings and in the schizonts. Labeling of the protein extracts from *P. yoelii* detected a 70 kDa polypeptide for the anti-747 antibodies in the sporozoites and the blood stages and a 60 kDa polypeptide for the anti-772 antibodies, only detected in *P. yoelii* sporozoites.

Further, to confirm the presence of proteins and their constancy of expression on the sporozoite surface deriving from several different parasites, we examined, by IFI, batches of sporozoites deriving from different Thai isolates of *Plasmodium falciparum*. The anti-772 serum had labeled all sporozoites, while only 7 out of 10 of the test isolates were labeled with anti-747. Similarly, PCR amplifications with primers specific for the two gene fragments (indicated in Figures 3.1a and 3.1b) were carried out with DNA from the blood stages of 12 different strains of *Plasmodium falciparum* (Figure 3.4). The PCR products corresponding to DG772 were amplified from 12 samples and their size was similar, while primers specific for DG747 could only amplify a fragment from 9 of the 12 DNAs. It should be pointed out that all of the parasite lines used in this study (T9-96, NF54 and 3D7) contain the corresponding genes. These results indicate a variation in the level of expression or the presence of DG747 in parasitic strains, as in total only 15 out of 22 parasites appeared to contain the DG747 gene or showed a positive reaction in IFI.

3.3. Recognition by the human immune system in endemic zones

In addition to the study regarding the constancy of expression of the antigen, we studied the prevalence of humoral responses of individuals living in highly (Dielmo) or weakly (Ndiop) endemic zones, and in two groups of different ages in these two zones (Figures 3.5a and 3.5b).

We observed the same prevalence (40%) against 747 in the weakly endemic zone, except that the number of individuals who responded strongly (intensity of response compared with controls) increased with age. In the highly endemic zone, the number of those responding against 747 increased with age, as well as the intensity of the response, and the prevalence in adults, who can be considered to have acquired immunity, was 85%. Further, these responses appear to correlate with exposure to the sporozoite, as the antibody count is higher in individuals

of a given age group in a stronger transmission zone. However, in a similar zone, the response did not change significantly during low transmission seasons (dry season) (results not shown) which could correspond to the response against the blood stages and/or indicate that the anti-747 immune response is long-term. The response induced by DG747 increased in prevalence and intensity as a function of exposure and the duration of exposure to the parasite (age).

The anti-772 response increased like anti-747 as a function of age, but with a much lower increase compared with the degree of transmission observed between Ndiop and Dielmo and compared with age. The degree of anti-772 responses, measured as a % prevalence and intensity, was higher for young individuals than for anti-747 responses, but lower in strength (75%) than the anti-747 (85%) in Dielmo in immune individuals.

3.4. Comparison of responses induced by different stages of the parasite

We had the advantage of being in possession of cells from individuals immunized with irradiated sporozoites, and serum from persons exposed to the parasitic infection in different manners.

3.4.1 Cell responses

All of the studies were carried out in close collaboration with Jean-Pierre Sauzet in the laboratory. Because of the small amount of material available, we restricted our analyses in order to detect what we had previously defined as one of the important criteria (role in protection) to evaluate a vaccinal potential in the pre-erythrocytic stage. We studied the secretion of IFN-γ from cells from 2 individuals immunized with irradiated sporozoites of *Plasmodium falciparum*, as we have observed, when analyzing other antigens, in particular LSA3 (a vaccine candidate studied in our laboratory (Daubersies et al, 2000)), that the degree of secretion of this cytokine appears to be correlated with protection. In these two volunteers, the number of cells secreting IFN-γ against DG747 and DG772 was as high as with recombinants from LSA3 (729 and PC), (Figure 3.6a). Further, we have examined whether the immune cell responses measured by proliferation of T lymphocytes and secretion of IFN-γ differed between

two chimpanzees immunized with sporozoites irradiated of *Plasmodium falciparum*, but one of which was not protected (Figure 3.6b).

Cells from the immune system of the animal immunized with viable irradiated sporozoites (18 kRad) and subsequently protected, recognized antigens DG747 and DG772, as did cells from the animal immunized with non-viable irradiated sporozoites (30 kRad), and not protected during a challenge infection.

Lymphocyte proliferation was at the limit of the threshold value, while the degree of secretion of IFN-γ was high, both for the quantity of cytokine detected and for the number of secreting cells (detected by Elispot). This was the case both for effectively immunized animals and for those which were not protected. However, it appears that the response levels were greater for animals immunized with sporozoites irradiated at 30 kRad. The responses induced by 747 were stronger than those induced by 772, and both were stronger than those induced by LSA3.

Cells removed from animals that have undergone supplemental immunization by irradiated sporozoites were damaged during transport from the primatology center in Africa, and thus we could not study the presence of a "boost" induced against said antigens.

3.4.2. Study of humoral responses

We were not in possession of cells from all human groups exposed in a different manner to parasitic infection, but we could study in detail the humoral response (IgG isotypes) from volunteers immunized with irradiated sporozoites (ISS, only exposed to pre-erythrocytic stages), from naturally immune individuals living in a highly endemic zone (exposed to all stages of the parasite), and from an individual having accidentally been infected with malaria by blood transfusion (only exposed to blood stages) (Figures 3.7a and 3.7b).

For the two antigens, the biggest difference was observed for the cytophilic isotype IgG1 the amount of which was much higher in serum from immune individuals (SHI) than in serum from a patient infected by transfusion or ISS volunteers. The responses from these two last

groups were fairly similar and did not bring about an imbalance between cytophilic antibodies (IgG1 and IgG3) and non cytophilic antibodies (IgG2 and IgG4). We also noted that the serum from an individual exposed for a long time to the parasite, but under permanent prophylaxis (PM), has the same profile of isotypes as the ISS.

3.5. Immunogenicity in the mouse

Mice from two different strains were immunized with recombinants in the form of proteins, with different adjuvants or in the form of "naked" DNA constructions, without adjuvant.

A preliminary study with the naked DNA construction comprising no signal sequence allowing export of the synthesized protein was carried out. The immunized mice generated no humoral response, whether for the two antigens of for other study simultaneously. However, we detected specific anti-747 and anti-772 cell responses. Both T lymphocyte proliferation and the degree of IFN-γ secretion were tested for the two mouse strains, C3H and BALB/c. The response profiles are shown in Tables 3.1a and 3.1b which show that for the case of cell responses from mice immunized with pNAK747, there was both proliferation and IFN-γ secretion stimulation, while for pNAK772, T cell proliferation was only slightly stimulated compared with stimulation of IFN-γ secretion which was considerable. Among all immunized mice, the highest level of IFN-γ secretion was observed when the level of proliferation stimulation was lowest.

Vaccinations with other formulations (recombinant protein and naked DNA with a signal sequence) have both induced a humoral response in the mice (Figure 3.8). All of the serum from said immunized mice recognized the native protein in IFI tests and the labeling corresponded with that observed for immunopurified human antibodies.

The anti-747 responses have a similar profile for all immunized mice and all of the formulations used, with an isotype response with IgG2b preponderance. The anti-772 responses were also similar between the mouse and vaccine formulations, but with a clear predominance of

IgG1. The isotype profile thus depends on the immunogen rather than on the mode of presentation employed. However, the end point titers were much higher when we immunized with recombinant proteins (1/200000) compared with DNA (1/2000), and the titers from the serum of mice immunized with His_6-772, were higher than those with His_6-747.

Since we observed a cross reactivity with the sporozoite stage of *P. yoelii*, we tested the protective potential of these antigens by infecting mice immunized with recombinant proteins with sporozoites from that species. Parasitemia was monitored by observation of the blood forms on smears from day 3 of the infection and for 12 consecutive days. We observed no protection regardless of the mouse strain employed, as parasitemia was detected on the same day as that for non immunized mice, and the graph was similar to that for control mice (results not shown).

3.6. Supplemental data

3.6.1. DG772

Using RT-PCR on the total RNA from sporozoites and blood parasites, the inventors could determine the splicing sites for the messenger RNA corresponding to the coding gene. The primer sequence was extracted from the genome data of *Plasmodium falciparum*.

The amplification products had identical sizes in the sporozoite stages and in the blood stages, and differed from the size of the product obtained by amplification of genomic DNA, and sequencing the splicing sites showed that they were identical (see the introns indicated in the Figure).

The gene coding for DG772 belonged to a family of proteins identified by a shared motif. All the proteins from the EBP (Erythrocyte Binding Proteins) family share conserved motifs from cysteine residues the arrangement of which is similar for all the proteins. However, the degree of identity does not exceed 31% (max 57% homology), even in the most highly conserved regions.

3.6.2. Immunogenicity tests envisaged in humans

From our results obtained in mice, we can confirm that the antigens DG747 and DG772, employed both in the form of DNA and in the form of recombinant protein, are immunogenic. Further, because the recombinant proteins are recognized by immunized individuals and protected against infection by *Plasmodium falciparum* sporozoites, it indicates a role for those antigens in pre-erythrocytic immunity. A test in primates and in particular in chimpanzees could allow the optimum formulation for clinical tests in human beings to be selected.

In phase I trials, to study the immunogenicity and safety of the product, we envisage three formulations, all prepared under GMP conditions: 1) the antigen in the form of a recombinant protein purified from the *Lactococcuc lactis* bacterium (use permitted in humans) supplemented with SBAS2 (GSK) adjuvant; 2) the DNA construct in the Vical^{TM} vector (Avantis Pasteur); and 3) synthetic lipopeptides injected without any adjuvant (see LSA3). Those formulations would be distributed by subcutaneous injection (into the deltoid). Our preliminary tests show that the antigens His_6-747 and His_6-772 induce cell responses and humoral responses in individuals who have only been exposed to the pre-erythrocytic stages. We would then study the cell responses and humoral responses in individuals immunized by the selected formulations by comparing them with those observed in individuals immunized with sporozoites attenuated by irradiation and protected against a challenge infection by non-attenuated sporozoites. Depending on those responses, a challenge infection with *Plasmodium falciparum* sporozoites would be envisaged.

3.6.3. Homology with other nucleotide sequences

Southern blot hybridization under stringent standard conditions (0.1 X SSC, 60°C) only gave rise to hybridization with the corresponding gene.

3.6.4. Identification of homology using bioinformatics means

Research was carried out using BLAST^{TM} software (tblastx and blastn) using all available databases for the *Plasmodium falciparum* genome and databases for other organisms. The parameters used were the default parameters found at http://www.ncbi.nlm.nih.gov/BLAST/.

3.7. Discussion

This work, which forms part of a study of antigens expressed in the pre-erythrocytic stages, allowed us to provide an initial characterization and evaluation of the vaccine potential of two novel *Plasmodium falciparum* antigens. These two antigens have different characteristics on the molecular level. Firstly, the protein of which DG747 forms a part contains repeats, while the molecule containing DG772 has not direct repeat. The Pfb0155c gene coding for DG747 is small (1524bp) and contains a repeat region to which DG747 belongs. We could not detect the presence of the gene by PCR in all strains, nor observe reactivity with all of the sporozoite strains studied. The observed absence could be due to a genuine gene deletion or to the experimental procedures. One of the primers used to detect the portion coding for DG747 crosses the repeat portion, which could cause difficulties in amplifying a gene containing a larger repeat, or in detecting a gene containing fewer repeats. Further, this is also the case when using indirect immunofluorescence detection, or any number of variations in the repeats may change the affinity of the specific antibodies, if the target epitope crosses that region. The expression detected by IFI appears to be present throughout the asexual parasitic cycle in the vertebrate host (we have not analyzed the sexual stages). Despite the presence of repeats in DG747, we have not detected cross reactivity with other *Plasmodium falciparum* antigens. The entire gene sequence was not homologous with other plasmodial proteins identified up to now, and we also had no indications of any biological function.

DG772 contains no repeats and its presence appears to be constant, whether detected by PCR or by IFI. On the biological level, the gene coding for DG772 appears to be interesting. We have found, by sequence homology, that this gene of 5300 bp with an open reading frame forms part of the EBP (Erythrocytic Binding Protein) family (Adams et al, 1992), but the sequence of DG772 does not belong to the conserved regions of that family; it shares only a small portion of sequences with the N-terminal end of the 3'cys region. Further, there are no cross reactivities nor sequence homologies with DG249, a further clone forming part of one of the consensus portions of the gene coding for EBA-175. It may be that DG772 forms part of a

region that confers particularity on each molecule of this family. The presence of two molecules from the EBP family (EBA-175 and 772) on sporozoites could imply that several molecules of this family exist could be alternatively involved in the invasion process as described in the blood stages.

A knowledge of prevalence is useful when evaluating a vaccine candidate, and the prevalences obtained for DG747 and DG772, 85% and 75%, are high. This study has shown the high antigenicity of a small portion of two molecules, and has suggested that it would be interesting to study other epitopes from the same molecule in more detail. The humoral response detected against DG747 and DG772 in permanently exposed individuals indicates that there is a preponderance of the IgG1 response (cytophilic type) developed during sustained exposure which is not found in the case of transfusional malaria. In contrast, profiles of humoral responses obtained for two groups exposed to the pre-erythrocytic stages of shorter or longer duration (PM and ISS) are similar and the IgG1 level is low, which indicates that this isotype results from repeated exposure to the antigen in the blood stages. A study of cell responses in these same zones should be carried out to obtain a more precise idea of the immune responses induced by those antigens.

At the pre-erythrocytic stages, we observed an induction of the cell response by these antigens both in chimpanzees and in humans. We have observed the secretion of IFN-γ, described as a factor involved in protection against pre-erythrocytic stages. The difference in response observed in chimpanzees as a function of the irradiation dose could mean that the antigens are recognized both on sporozoites and in the hepatic stages. Sporozoites irradiated at 30 kRad are incapable of penetrating into the hepatocyte, and the detected responses are thus only due to that exposure, while sporozoites irradiated at 18 kRad develop in the hepatocyte, and the detected responses are thus due to this stage. It would be interesting to study more closely the responses induced in these two animals (MHC1 restriction), and the response "boost"

induced during several successive immunizations. This work has also shown that in these two antigen fragments, T epitopes and B epitopes both exist.

The immunogenicity induced by the two antigens in the mouse is different, with an IgG1 predominance for DG772, which is not observed for DG747. In contrast, we have not observed any differences in response as a function of formulations, which is interesting as the presentation of the molecules is not identical for each formulation. The cell responses obtained only for the formulation that did not induce detectable humoral responses shows that there was both lymphocyte proliferation and IFNγ secretion, dependent on the mouse.

Table D1: Summary of pre-erythrocytic malaria antigens known at the present day*

Antigen	References[1]	Expression stages[2]				Location[3]	Structures	
		S	H	SSA	SSS		Intron	Reps
CS	Nardin et al 1982	+	j	-	-	CHR3	+	+
LSA1	Guérin-Marchand et al 1987	+	j,m	-	-	CHR4	-	+
TRAP	Robson et al 1988	+	j,m	m	-	CHR13	-	+
PfHsp70	Renia et al 1990	+	j,m	j,m	-	CHR13		+
STARP	Fidock et al 1994	+	j,m	-	-	CHR12	+	+
EXP-1	Koenen et al 1984 Sanchez et al 1994	-	m	j,m	Nt	CHR12	-	+
Pfs16	Bruce et al 1990	+	nt	-	+	?	-	-
SALSA	Bottius et al 1996	+	j,m	-	-	CHR2	+	-
LSA3	Daubersies et al 2000	+	j,m	-	-	CHR2	+	+
PfEMP3	Pasloske et al 1993 Grüner et al 2001	+	j,m	j,m	-	CHR2	+	+
GLURP	Borre et al 1991	+	m	j,**m**	Nt	CHR10	-	+
EBA-175	Camus and Hadley, 1985	+	j,m	j,m	-	CHR4,13 ?	+	-
DG747		+	j,m	j,**m**	-	CHR2	?	+
DG772		+	j,m	j,m	-	CHR1	+	-

* The antigens shaded in gray are the antigens characterized in the present application.

NT: not tested

1: The presence of other antigens (MSP-1) in the pre-erythrocytic stages has also been suggested, but the preliminary results still have to be confirmed. The underscored references indicate the year in which pre-erythrocytic expression was discovered.

2: S: sporozoite; H: hepatic stage, young and mature; SSA: asexual blood stage, young and mature; SSS sexual blood stage. 3. ST: sub-telomeric. The bold characters indicate the form of the stage in which labeling is the most intense.

3: The majority of chromosomal location detection was carried out by homology identification using databases.

Table 3.1a: Stimulation of cell proliferation and secretion of IFN-γ by His_6-747 after 3 immunizations with pNAK747

Mouse strain	Lymphocyte proliferation stimulation index		Gamma interferon IU/ml	
	His_6-747	pGEX-NN	His_6-747	pGEX-NN
C3H	**8.6 ± 3.0**	3.2 ± 1.1	7.0 ± 0.4	4.0 ± 0.9
C3H	**23.6 ± 5.9**	8.8 ± 2.9	7.0 ± 0.7	4.0 ± 0.2
C3H	3.0 ± 0.9	1.1 ± 0.1	**16.0 ± 1.9**	1.0 ± 2.1
Positive	**2/3**	-	**1/3**	-
BALB/c	2.7 ± 0.2	1.3 ± 0.2	**40.0 ± 5.0**	24.0 ± 3.0
BALB/c	**23.6 ± 4.3**	3.0 ± 0.3	**15.0 ± 4.4**	8.0 ± 1.8
BALB/c	**33.7 ± 7.2**	5.9 ± 0.3	**16.0 ± 1.5**	10.0 ± 4.2
Positive	**2/3**	-	**3/3**	-

pGEX-NN: *Plasmodium falciparum* antigen, not cross-reactive with His_6-747.
Positive results are shown in bold.

Table 3.1b: Stimulation of cell proliferation and secretion of IFN-γ by His_6-772 after 3 immunizations with pNAK772

Mouse strain	Lymphocyte proliferation stimulation index		Gamma interferon IU/ml	
	His_6-772	pGEX-NN	His_6-772	pGEX-NN
C3H	0.9 ± 0.1	0.7 ± 0.1	**23.9 ± 2.0**	10.7 ± 2.9
C3H	0.9 ± 0.2	0.6 ± 0.1	1.7 ± 2.0	3.3 ± 2.1
C3H	0.8 ± 0.2	0.9 ± 0.2	6.6 ± 0.1	5.8 ± 2.6
Positive	**0/3**	-	**1/3**	-
BALB/c	**3.5 ± 0.3**	2.7 ± 0.4	**24.0 ± 2.2**	10.8 ± 3.0
BALB/c	1.9 ± 0.3	1.2 ± 0.4	**31.3 ± 7.1**	5.6 ± 0.9
BALB/c	**3.2 ± 0.9**	1.5 ± 0.2	**25.0 ± 12.1**	6.9 ± 1.5
Positive	**1/3**	-	**3/3**	-

pGEX-NN: *Plasmodium falciparum* antigen, not cross-reactive with His_6-772.
Positive results are shown in bold.

Table 4.1: Cell responses in mice after 5 immunizations with pNAK438*

Mouse	Proliferation (IS)	IFN-γ secretion	Antibodies
C3H	**2.3 ± 0.3**	2.2 ± 2.0	-
C3H	1.8 ± 1.1	**15.6 ± 4.0**	-
C3H	**3.2 ± 1.3**	0.5 ± 0.2	-
Positive	**2/3**	**1/3**	
BALB/c	**4.3 ± 1.6**	**26.2 ± 6.3**	-
BALB/c	**5.3 ± 0.6**	**29.3 ± 8.5**	-
BALB/c	**15.3 ± 2.2**	**12.7 ± 8.7**	-
Positive	**3/3**	**3/3**	

* The level of responses is shown with respect to the responses obtained by a threshold value. The threshold value was calculated by taking the mean of the responses of non-immunized animals and that of animals immunized against a non-relevant antigen such as OspC, a protein from *Borrelia burgdorferi*.

Table 4.2: Detection of expression by IFI with immunopurified human antibodies or specific anti-His$_6$-680 mouse serum

Parasites*	a-His$_6$-680 mouse or human
Plasmodium falciparum NF54 sporozoites *P. yoelii* clone 1.1 sporozoites	++ ++
Plasmodium falciparum hepatic stage	++
Blood stage T23 rings/schizonts NF54 blood stage rings/schizonts	++/+++ (75%) ++/+++ (75%)

* *Plasmodium falciparum* sporozoites from NF54 strain.
T23:strain of Thai provenance; NF54: strain of African origin.

Table 5.1: Cross reactivities detected in Western blots between members of the Pf11-1 family

	P43	P263	P322	P453	P525	P563	P571
E43							
E263							
E322							
E453							
E525							
E563							
E571							
E676f							
E571							

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Strong reaction

Medium reaction

E: immunopurified antibodies (eluted), on corresponding recombinant proteins.

P: recombinant protein.

Table 5.2: Cross reactivities on nucleotide level between clones of the Pf11-1 family*

PM clones	PCR571 (1)	Control probe
43	+++	++
88	++	0
322	+++++	+++++
525	++++	+++++
563	++++	++++
571	+++++	+++++
676f	+++++	++++
729E	+++	+++++
263	++++	NT
381	++	NT
453	+++	NT

*Signal intensity symbolized by plus signs.

NT: not tested

Table 5.3: Homologies of clone sequences studied by BLAST

Clone	Nucelotide size (bp)[1]	Amino acid repeats	Homology Nucleotides **Proteins**	Degree of homology with 571 Nucleotides **Proteins**
DG43	900	PIVeELLEE	Pf11-1 Part 1: 94%, **100%** (= 88)	80%, **80%**
DG88	900	PIVeELLEE	Pf11-1 Part 1: 94%, **100%** (= 43)	Idem DG43
DG263-7	253	None	*Plasmodium falciparum* Chr 12 95%, **60%**	-
DG263-8	176	None	*P.f.* CHR12 95%, **45%**, human chr22 **58%**	-
DG322-1	500	-	-	-
DG322-2	**2000**	PeeVLEEvl	Pf11-1 86%, **65%**	79%
DG381	400	PEklvEEVI	plastid tRNA 100%, **CHR2 71%**	-
DG453	300	PIVEEvVEE	Pf11-1 Part 2 88%, **83%**	93%, **72%**
DG525	450	PeleEVEvl	GLURP R2 98%, **100%**	-
DG563	438	PIVEEvvEE	Pf11-1 Part 4: 86%, **Part 1 68%**	75%, **56%**
DG571	550	PEEiIEEiv	Pf11-1 Part 5 87%, **55%**	100%, **100%**
DG676f	2000	PvVEEvLEE	Pf11.1 Part 4 88%, **Part 5 75%**	74%, **44%**
DG729E	1.7	-	mal3P5 100%	-

1: Estimated size with respect to PCR products obtained, or the precise size when the entire clone has been sequenced.

CHR: chromosome; part: portion

Table 5.4: IFI reactivity tested with antibodies specific to His_6-571 and Vi571

Parasites	a-His_6-571, aVi571 mouse or human
Plasmodium falciparum NF54 sporozoites	++
P. yoelli clone 1.1 sporozoites	++
Plasmodium falciparum hepatic stage	++
T23 blood stage rings/schizonts	++/+++ (75%)
NF54 blood stage rings/schizonts	++/+++ (75%)

Table 5.5: Cell response in mice immunized with pNAK571

Mouse strain	Lymphocyte proliferation stimulation index		Gamma interferon IU/ml	
	571 pGEX	NNpGEX	571 pGEX	NNpGEX
C3H dead	0.9 ± 0.1	0.7 ± 0.05	31.0 ± 2.5	21.21 ± 10.6
C3H	0.9 ± 0.2	0.7 ± 0.1	**30.2 ± 2.6**	10.50 ± 2.03
C3H	0.8 ± 0.2	0.9 ± 0.2	**16.8 ± 0.1**	6.65 ± 2.8
Positive	**0/3**	-	**2/3**	-
BALB/c	**3.5 ± 0.3**	2.7 ± 0.13	**23.1 ± 0.9**	10.36 ± 2.2
BALB/c	1.9 ± 0.3	1.2 ± 0.1	**15.9 ± 2.1**	8.63 ± 3.9
BALB/c (dead)	**3.2 ± 0.9**	1.5 ± 0.1	**5.3 ± 1.3**	1.06 ± 0.5
Positive	**2/3**	-	**3/3**	-

NNpGEX: GST fusion recombinant of non relevant LSA3.

Although the present invention has been described with respect to preferred implementations, it would be clear to persons skilled in the art or science in question that it would be possible to introduce variations and modifications without departing from the scope of the invention described and claimed in this document.